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PBMC processing and stimulations

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An abbreviated version of this protocol was published in Science Advances in Nov 2020

Single-cell peripheral immunoprofiling of Alzheimer's and Parkinson's diseases

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Detailed protocol

PBMC Isolation Process

By Rosemary Fernandez

Materials:

- All pipet sizes + tips
- Genemate pipettor
- 10 mL stripettes
- 15 & 50 mL Falcon tubes
- Ficoll-Paque PLUS (sold as 6 x 100 ml by GE Healthcare)
- PBS pH 7.4 (1x) by Life Technologies
- Pipette with dropwise/slow setting
- Cryovials
- Freezing media A: FBS
- Freezing media B: 20% DMSO solution in FBS
- Propidium iodide
- Hemocytometer/MoxiFlow
- Microscope
- Room temperature Mr. Frosty

Procedures:

1) Defrost Mr. Frosty if it is not at room temperature.
2) Transfer all of the blood into a 50 mL Falcon tube.
3) Pour 1 mL PBS into original tube containing the blood. Rinse sides of tube to get all of the remaining blood, and pour this into the 50 mL Falcon tube with the rest of the sample.
4) Add PBS to the 50 mL Falcon tube (1:1 ratio of blood to PBS)
5) In a separate 50 mL Falcon tube, add 15 mL of Ficoll-Paque PLUS.
6) Set your pipette setting to low (preferably drop-wise). Add the blood solution to the Ficoll-Paque tube slowly to separate out the layers. This is done by tipping the Falcon tube with the Ficoll-Paque to a 45-degree angle and smearing the blood along the side of the tube.

(NOTE: Add only 15-20 mL of blood above the Ficoll solution. If you have more than 15-20 mL of blood, use more Falcon tubes with Ficoll)

7) Centrifuge between 18-20*C for 30 min. (1200x g) without brakes
8) Discard the upper layer of plasma and collect the white layer of cells and place into a new 50 mL Falcon Tube. Make sure not to collect any of the underlying Ficoll layer.
9) Add PBS up to the 50 mL mark for the first wash.
10) Centrifuge at 1650 rpm (575 x g) for 10 min.
11) Remove supernatant and resuspend the pellet by tapping the tube.
12)Add 0.5 ml of Freezing Media A (FBS)(for 6-10 ml whole blood) to the pellet and mix.
13)Remove 10 ul of cells and mix with 10 ul Trypan Blue to do a cell count on the hemocytometer.

14) Based off the total cell count, calculate the number of vials and volume of freezing media that will be needed.

15)Dropwise add 0.5 mL Freezing media B (80 % FBS+ 20 % DMSO) and carefully mix the cells.

16) Resuspend cells in enough freezing medium B such that the DMSO is at final 10% to create a cell suspension of 10 million per ml. Pipette up and down to ensure even mixture and aliquot about 1ml into storage vials. This will provide 10 million cells per cryovial.

17)Label cryovials (on top of cap and on the side with permanent alcohol/waterproof marker):
PIDN
DATE
PBMC
Volume
18) Place cryovials (polypropelene) in room temp Mr. Frosty (check if isopropanol level is good).
19) Place Mr. Frosty in the -80℃ freezer for 24 hours.
20) Transfer cryovials to liquid nitrogen tank the next day for long term storage.
21) Log into database

PBMC Stimulation Process

By Rosemary Fernandez

Materials and Reagents
1. PBMC (fresh or thawed frozen)
2. Benzonase (Pierce Antibodies, catalog number: 88701)
3. Cytokine aliquots (IFNα, IFNγ, IL-6, IL-7, IL-10, IL-21, IL-2 etc.)
 a. IFNa (PBL Interferon source, catalog number: 11105–1)
 b. IFNg2 (BD Biosciences, catalog number: 554617)
 c. IL6 (BD Biosciences, catalog number: 550071)
 d. IL7 (BD Biosciences, catalog number: 554608)
 e. IL10 (BD Biosciences, catalog number: 554611)
 f. IL21 (Life Technologies, Gibco®, catalog number: PHC0214)
 g. IL2 (BD Biosciences, catalog number: 554603)
 h. CD3 (BD Biosciences, catalog number: 555329)
 i. CD28 (BD Biosciences, catalog number: 555725)
 j. LPS (Sigma-Aldrich, catalog number: L7770)
 k. IL5 (Pepro Tech, catalog number: 200–05)
 l. IL17A (Pepro Tech, catalog number: 200–17)
4. 16% PFA (Alfa Aesar, catalog number: 4368)
5. Methanol (Thermo Fisher Scientific, catalog number: A452SK-1)
6. Deep Well plate (Costar, catalog number: 3960)
7. Phenotyping and phosphoprotein antibodies filtered with 0.1 um spin filters (EMD Millipore, model: UFC30VV00)
8. Ir-intercalator stock solution from DVS (Rh103-intercalator can be used) (catalog number: 201192 B)
9. 1x CyPBS PBS (Rockland, catalog number: MB-008)
10. Complete RPMI (see Recipes)
11. CyFACS buffer (see Recipes)

Equipment
1. 37 °C water bath
2. Biosafety cabinet
3. Centrifuge
4. CO_2 incubator at 37 °C
5. Calibrated pipettes
6. 8 or 12 pin aspirator (V&P Scientific, model: VP187A)

Procedure
A. Thaw PBMC
1. Warm complete RPMI to 37 °C in water bath. Each sample will require 20 ml of complete RPMI with benzonase to limit cell clumping. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm media with 1:10,000 benzonase (final concentration 25 U/ml). Remove samples from liquid nitrogen and transport to lab on dry ice.
2. Place 10 ml of warmed benzonase media into a 15 ml tube, making a separate tube for each sample.
3. Thaw frozen vials in 37 °C water bath.
4. When cells are partially thawed, carry to hood.
5. Add 1 ml of warm benzonase media from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more media from centrifuge tube to retrieve all cells.
6. Continue with the rest of the samples as quickly as possible.
7. Centrifuge cells at 1,600 rpm (RCF = 390) for 10 min at room temperature.
8. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
9. Gently resuspend the pellet in 1 ml warmed benzonase media. Centrifuge cells at 1,600 rpm (RCF = 390) for 10 min at room temperature. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
10. Resuspend cells in 1 ml warm complete RPMI.
11. Count cells with Vicell (Vi-Cell XR Beckman Coulter) (or hemocytometer if necessary). To count, take 20 µl cells and dilute with 480 µl PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.
12. Adjust the cell concentration to 5 × 106 cells/ml with warm media (no more benzonase at this point.)
13. Using a multichannel pipette, add 100 µl cells (0.5 × 106 cells) into each of eight wells of a 96-well deep well plate.
14. Rest for another 1 h–1.5 h at 37 °C in CO2 incubator. Prepare the stimulation plate just before stimulation.

B. Stimulate cells
1. Prepare cytokines at 5x concentrations, in 500 µl of RPMI in a fresh deep well block.
2. Add enough 5x cytokine (~500 µl for a full plate) into one row of a fresh deep well block to pipette

2. Add enough 5x cytokine (~300 µl for a full plate) into one row of a fresh deep well block to pipette.

Example of cytokine stimulation:

IFNa: Final concentration of stimulation used= 10,000 units/ml

IFNg2, IL6, IL7, IL10, IL21, IL2, IL17A: Final concentration of stimulation used= 50 ng/ ml

CD3 = 2.5 µl in 990 µl (final concentration 500 ng/ml)

CD28 = 10 µl in above media final (concentration 2,000 ng / ml)

LPS: Final concentration of stimulation used= 1 µg/ ml

PMA 10 ng/ml final concentration ml

Ionomycin 1,000 ng/ml final concentration /ml

IL5: Final concentration of stimulation used= 10 ng/ml

3. Remove rested cells in the deep well block from incubator and stimulate by adding 25 µl of 5x cytokines with multichannel pipette to each row of patient samples. Change tips between each patient. Work as rapidly as possible.
4. Tap plate to mix, and incubate 15 min at 37 °C in CO2 incubator.
5. Remove cells from incubator at the 15 min and using a multichannel pipette, add 20 µl 16% PFA (~2.2% PFA final concentration) to each row of patient samples in the deep well block. Pipette up and down to mix for each patient. Change tips between patients. Add PFA in the same order that you added the cytokine stimulation.
6. Incubate 10 min at room temperature.
7. Add 1.6 ml CyFACS buffer to each well of the deep well block.
8. Centrifuge cells at 974 rcf (x g) for 10 min at 4 °C. The cells are fixed / dead and has to be spun down harder to prevent cell loss.
9. Aspirate supernatant from the cells.

C. Surface staining

1. Make cocktail in PBS of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Make sufficient volume for each sample to have 20 µl of cocktail. Pipet into 0.1 µm spin filter and centrifuge in a tabletop microcentrifuge (RCF = 14,000) for 5 min at room temperature.
2. Add 20 µl of antibody cocktail to the cells in the deep well plate, vortex to mix and let it incubate at RT for 30 min.
3. Wash cells in 1.6 ml/well x2 with CyFACS buffer and centrifuge cells at 974 rcf (x g) for 8 min at 4 °C. Discard supernatant by aspiration.
4. Permeabilize the cells by adding 600 µl –20 °C cold MeOH to each well of the deep well block using a multichannel pipette. Pipette up and down to mix for each patient. Change tips between patients.
5. Cells are stored overnight at this point at −80 °C.
6. Remove samples from freezer.
7. Wash in 1 ml/well CyFACS buffer.
8. Centrifuge cells at 974 rcf (x g) for 10 min at 4 °C. Discard supernatant by aspiration.
9. Wash in 1.8 ml / well CyFACS buffer.
10. Centrifuge cells at 974 rcf (x g) for 10 min at 4 °C. Discard supernatant by aspiration.

D. Intracellular staining

1. Make cocktail in PBS of metal-chelating polymer-labeled intracellular antibodies according to previously determined titration. Make sufficient volume for each sample to have 20 µl of cocktail. Pipet into 0.1 µm spin filter and centrifuge in a tabletop microcentrifuge (RCF = 14,000) for 5 min at room temperature.
2. Add 20 µl of antibody cocktail to the cells in the deep well plate and let it incubate at RT for 30 min.
3. Wash in 1.8 ml/ well CyPBS.
4. Centrifuge cells at 974 rcf (x g) for 10 min at 4 °C. Discard supernatant by aspiration.
5. Make 1:200 dilution in CyPBS of Ir-intercalator. Add 20 µl of diluted Ir-intercalator solution to each well, pipet to mix. Incubate on ice for 20 min.
6. Wash 1.6 ml/ well x 3 in MilliQ water.
7. Centrifuge cells at 974 rcf (x g) for 10 min at 4 °C. Discard supernatant by aspiration.
8. Acquire samples on the CyTOF, after standard instrument setup procedures.

Recipes

1.Complete RPMI (Hyclone RPMI-1640 Medium)

RPMI with 10% FBS

Pencillin/streptomycin

Glutamine

2.CyFACS buffer

1x CyPBS PBS with 0.1% BSA, and 0.05% Na azide

Made in MilliQ water

Note: Do not use FBS!

References

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2. Rosemary Fernandez and Holden Maecker. Cytokine-Stimulated Phosphoflow of PBMC using CyTOF mass cytometry. Bio Protoc 2015

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1. Phongpreecha, T. , Fernandez, R. and Montine, T. (2020). PBMC processing and stimulations. Bio-protocol Preprint. bio-protocol.org/prep721.
2. Phongpreecha, T., Fernandez, R., Mrdjen, D., Culos, A., Gajera, C. R., Wawro, A. M., Stanley, N., Gaudilliere, B., Poston, K. L., Aghaeepour, N. and Montine, T. J.(2020). Single-cell peripheral immunoprofiling of Alzheimer's and Parkinson's diseases. Science Advances 6(48). DOI: 10.1126/sciadv.abd5575

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